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INTERACTION OF IONIZED AND UN-IONIZED AMMONIA ON SHORT-TERM SURVIVAL AND GROWTH OF PRAWN LARVAE, *MACROBRACHIUM ROSENBERGII*

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Ammonia is the principal excretory product of Crustacea (Hartenstein, 1970; Hochachka and Somero, 1973; Kinne, 1976), and its modes of toxicity as well as concentrations lethal to a variety of organisms have been well documented (Warren, 1962; Campbell, 1973). Ammonia exists in solution primarily as the NH_4^+ ion and the un-ionized NH_3 molecule, the proportions of which are highly pH-dependent. In this paper *ammonia* will refer to the sum of NH_4^+ and NH_3. *Un-ionized ammonia* will refer to the NH_3 molecule and *ionized ammonia* to the NH_4^+ form.

In the aquatic habitat, organisms rely on rapid diffusion of NH_3 across the gill membranes (Fromm and Gillette, 1968) or exchange transport of NH_4^+ with Na^+ (Maetz and Garcia-Romeu, 1964; Campbell, 1973; Mangum and Towle, 1977) to void themselves of this toxicant. Diffusion of NH_3 is a principal route of excretion because blood levels are normally much greater than ambient concentrations (see Kinne, 1976, for review). Fromm and Gillette (1968) reported that ammonia levels in the blood of trout are 9–40 times greater than in ambient water. Concentrations of ammonia in the blood of Crustacea range from 2 to 18 mg/liter (Myers, 1920; Florkin and Renwart, 1939; Mangum, Silverthorn, Harris, Towle and Krall, 1976), which are one to several orders of magnitude greater than concentrations in their habitat (Kinne, 1976). As external NH_3 concentrations increase, the rate of diffusion outward from an animal decreases and toxicity ensues when tolerable body loads are exceeded. Consequently, the toxicity of ammonia to aquatic organisms is generally credited to the NH_3 molecule (Ellis, 1937; Wuhrmann and Workers, 1948; Downing and Merkens, 1955; Spotte, 1970; Hampson, 1976), despite evidence that NH_4^+ adversely affects some physiological functions (Shaw, 1960; Maetz, 1972; Campbell, 1973).

The chemistry of ammonia in solution has been discussed by Whitfield (1974) and Colt and Tchobanoglous (1976). The proportion of total ammonia existing as NH_3 is dependent on temperature and ionic strength of the medium, but primarily on the pH of the solution (Warren, 1962; Trussell, 1972; Skarheim, 1973; Whitfield, 1974; Emerson, Russo, Lund and Thurston, 1975). Calculations by these authors show that the NH_3 fraction of ammonia increases as pH rises; an increase of one pH unit elevates the NH_3 concentration tenfold. As previously stated hypotheses have suggested, the toxicity of an ammonia solution should increase at higher pH values.

There has been little work done on the sensitivity of crustaceans to ammonia poisoning. During the course of this study only two reports were found which give

systematic evaluations of ammonia toxicity (based on mortality) to Crustacea (Anderson, 1944; Wickins, 1976), and a single study investigating the sensitivity of larval crustaceans to ammonia (Delistraty, Carlberg, Van Olst and Ford, 1977). Adverse effects of ambient ammonia on some physiological functions have also been reported by Shaw (1960), who found a significant reduction in sodium influx in the crayfish *Astacus pallipes*, and by Mangum *et al.* (1976), who reported reduced ammonia excretion rates in the blue crab, *Callinectes sapidus*. The exposure levels of ammonia in these experiments were high, 18 and 180 mg NH_4^+/liter, respectively, and may have been approaching lethal concentrations. However, the authors' interests were in impairment of physiological functions, and gross signs of stress or mortalities at these concentrations were not discussed.

It seems, therefore, that most researchers investigating the effects of ammonia on organisms tend to concentrate on one molecular form or another in designing and analyzing their work. On the one hand, those interested in concentrations lethal to fish and crustaceans underline the importance of the NH_3 species because of its ease in diffusing across membranes. Consequently, toxic levels of up to a few mg NH_3/liter may represent well over 100 mg NH_4^+/liter, especially at pH < 8.0. Such high ammonium ion concentrations may well contribute to observed mortality and should not be ignored.

On the other hand, physiologists concerned with the interactions between $Na^+ - NH_4^+$ in salt transport processes fail to address, first, the possibility that the NH_3 portion of the high total ammonia concentrations used (Carrier and Evans, 1976; Mangum *et al.*, 1976; Towle, Palmer and Harris, 1976) may constitute a severe stress to an organism or cellular system, thereby affecting a process that is thought to be NH_4^+-mediated only; and secondly, the effect that NH_4^+ inhibition of Na^+ transport or ammonia excretion may have on survival of organisms in different habitats.

The following study was performed to determine: first, concentrations of ammonia lethal to larval *Macrobrachium rosenbergii* in short-term exposures; secondly, roles and interaction of NH_3 and NH_4^+ in affecting toxicity using pH as a variable, and to analyze any observed interaction in light of possible physiological mechanisms; and thirdly, sublethal effects during short-term exposure using growth-reduction as the criterion of toxicity. An additional motive underlying this study was to gain information on ammonia toxicity that could be applied to general water quality requirements for crustaceans. This is particularly important since ambient ammonia concentrations in culture or holding water may often exceed levels recommended as safe (Spotte, 1970) despite extensive filtration.

Materials and Methods

Animals

Larvae were produced by second generation U. C. Davis brood stock initially obtained from Hawaii and Thailand. Broods were hatched and mass-reared in 80 liter glass aquaria with water circulated through biological filters. Water temperature and salinity were 27–28° C and 12‰ (Instant Ocean salts). Larvae were fed newly hatched *Artemia salina* nauplii and were used in tests from three to eight days after hatching.

Lethal toxicity bioassays

Static bioassays to assess ammonia toxicity were performed as described by Armstrong, Stephenson and Knight (1976a), for nitrite toxicity experiments wtih *Macrobrachium*. Fifteen larvae were placed in each 250 beaker containing 200 ml of test solution; the ratio of dry weight animal biomass (shrimp + *Artemia*) to volume of solution ranged from 3 to 17.5 mg/liter. Ammonia concentrations were made by serial dilution of reagent grade NH_4Cl (Mallinckrodt) for a concentration range of 1.0–320 mg ammonia/liter, spaced in threefold increments per decade. All amonia concentrations and controls at each pH were replicated, and the experiment was run twice with two broods of larvae.

Test solutions were renewed every 24 hr at which time larvae were transferred to new beakers, mortalities recorded, and fresh brine shrimp added to give a density of about 4–6 nauplii/ml. After the initiation of an experiment, mortalities were checked at 30 min, 1, 2, 4, 8, 16, and 24 hr, and three times in each subsequent 24 hr interval to the conclusion of 144 hr. In the first 24 hr death was defined as the cessation of heart beat and pulsing of the posterior intestine. Thereafter, the opaqueness commonly developed by moribund and dead larvae was used as the criterion of death (Armstrong *et al.*, 1976a; Armstrong, Buchanan, Mallon, Caldwell, and Millemann, 1976b).

Test water was maintained at 28° C (all beakers held in a single water bath) and 12‰ salinity. The photoperiod was 9D: 15L. Three pH values tested were 6.8, 7.6, and 8.4. Stock water of 12‰ was held in 20 liter carboys, aerted and adjusted frequently to desired pH with 1 M NaOH or HCl until levels stabilized which required several days prior to a test. The pH of test solutions was checked three times/24 hr period and adjusted with 0.1 M NaOH or HCl. Solutions to which high ammonia concentrations were added (> 100 mg ammonia/liter) required pH adjustment immediately. A Corning model 12 pH meter with Ag/AgCl and calomel electrodes, standardized with NBS type buffers, was used for measurements. Some investigations suggest inaccuracies in measuring pH of high ionic strength solutions on meters calibrated with low ionic strength buffers. Hansson (1973) stated such error could be 0.09 pH units at 20‰, and Whitfield (cited in Wickins, 1976) reported a 0.05 unit error at 35‰. Since our salinity was 12‰, we do not consider the possible error due to calibration with NBS type buffers to be significant. The mean pH values (calculated by converting pH to hydrogen ion concentration, averaging and then returning the means and standard deviation to pH units) were 6.83 ± 0.09, 7.60 ± 0.09, 8.34 ± 0.06 ($n > 100$ for each pH). These values were used to calculate un-ionized ammonia concentrations.

Beakers were not aerated; yet dissolved oxygen, measured with a Beckman O_2 Analyzer, exceeded 90% of saturation (7.3 mg/liter at temperature and salinity used) after 24 hr in all pH and ammonia concentrations. High dissolved oxygen values were due to the change of solutions every 24 hr, low biomass to volume ratio, and our procedure of stirring the solution of beakers several times a day to check for deaths.

Nitrite was measured in representative concentrations from all three pH values by a sulfanilamide-based colorimetric reaction (Federal Water Pollution Control Administration, 1969). At the end of 24 hr, the average nitrite concentration was

9.4 ± 3.3 μg NO_2-N/liter, which is several orders of magnitude lower than the incipient lethal level of 3 mg NO_2-N/liter reported by Armstrong *et al.* (1976a) for *Macrobrachium*.

Ammonia was measured with an Orion Ammonia Electrode Model 95–10 coupled with the Corning pH meter. Merks (1975) states that this probe loses accuracy with increasing salinity, and correction factors must be used. However, we made ammonia standards with fresh water and 12‰ sea water and found no difference in millivolt readings for the same ammonia concentrations in the two media. The average ammonia concentation at 24 hr in control beakers of all pH values was 0.45 ± 0.11 mg ammonia/liter. By the end of a 24 hr period the change in ammonia levels in test beakers was minimal. Average measured concentrations were 102% of time-zero nominal levels, indicating little volatilization or nitrification of the chemical during tests.

Growth experiments

Larvae of this warm water species grow rapidly, molting and gaining substantial weight in five to seven days (George, 1969; Armstrong *et al.*, 1976a). Therefore, documentation of sublethal effects by studying growth seemed feasible during short-term exposures. After establishing lethal concentrations of ammonia at each pH, identical bioassays were performed using two sublethal concentrations per pH. A time-zero sample of 25 larvae was dried at 70° C for 24 hr. Animals were then individually weighed on a Cahn Model 4700 automatic electrobalance, accurate to a few μg. Test animals were exposed as previously outlined and at the conclusion of a test were dried and weighed individually. A relative growth rate was calculated for each treatment with the formula of Waldbauer (1968): $G_R = P/TM$, where P is mean dry weight gain between sampling period, T is time between sampling period and M is mean individual weight over the sampling period. Two sublethal growth experiments were done: the first with five-day old larvae exposed to treatments for five days; and the second with three-day old animals exposed for seven days.

Statistical analyses

The effect of ammonia concentration on survival was investigated using a three-way analysis of variance. The effects of brood, pH, concentration and their interactions on the dependent variable, time to death for each larva, were analyzed. Effects of sublethal concentrations on growth were investigated with one-way ANOVA, by treating each pH-concentration combination as a separate factor. If a significant F value ($P < 0.01$) was obtained, treatment differences were contrasted by means of a Q value (Snedecor and Cochran, 1967; these authors regard this contrasting procedure as a conservative gauge of true differences). LC_{50} values (the concentration of toxicant lethal to 50% of the test organisms in a specified time period) were derived from log-probit plots of concentration *vs.* mortality. LT_{50} values (the time required for death of 50% of the organisms in a given concentration of toxicant) were obtained by probit analysis program BMD/O3S (Dixon, 1970).

Calculation of un-ionized ammonia: NH_3

The NH_3 fraction of the total ammonia measured is calculated from the general formula for bases (Albert, 1973):

$$[NH_3] = \frac{[\text{Ammonia}]}{1 + 10^{(pKa - pH)}} \quad (1)$$

The measurement, and possible inaccuracies, of ammonia and pH have been discussed. The pKa remains as the major variable of the equation and is influenced by physical conditions of the solution. Emerson *et al.* (1975) found the temperature dependence of the pKa value to be:

$$pKa = 0.09018 + 2729.92/T \quad (2)$$

$$T = \text{degrees Kelvin}$$

Equation (1) is based on an infinite dilution model for which the activity of an ion approaches its analytical concentration as the solute concentration approaches zero. For freshwater systems such a model is accurate. However, as the solute concentration (*i.e.*, salinity) of a solution increases, the activity of ions and uncharged species may be significantly different from their concentration. In turn, such changes will affect pKa values and, in the case of equation (1), will consequently change the concentration of un-ionized ammonia calculated.

The pKa values of the ammonia system in sea water have not yet been experimentally determined. Whitfield (1974) developed theoretical pKa values for sea water, but did not calculate them for salinities less than about 20‰. The salinity of our tests was 12‰ (ionic strength, $I = 0.242$) for which an appropriate pKa value was derived.

The acid dissociation reaction for ammonia in water is:

$$NH_4^+ + nH_2O \rightleftharpoons NH_3 \cdot nH_2O + H^+ \quad (3)$$

The equilibrium expression for this reaction is:

$$Ka = \frac{\{H^+\}\{NH_3 \cdot nH_2O\}}{\{NH_4^+\}\{H_2O\}^n} \quad (4)$$

where Ka = acidity equilibrium constant

$\{i\}$ = activity of the ith species

Rewriting equation (4) partially in terms of concentration

$$Ka = \frac{[NH_3]\gamma_{NH_3}\{H^+\}}{[NH_4^+]\gamma_{NH_4^+} \cdot \{H_2O\}^n} \quad (5)$$

where $\{i\} = \gamma_i[i]$

$[i]$ = concentration of the ith species.

Since the electrode method for determining pH measures the activity of the hydrogen ion rather than concentration, it is convenient to retain the $\{H^+\}$ term. Re-

writing equation (5)

$$\frac{Ka \cdot \{H_2O\}^n \gamma_{NH_4^+}}{\gamma_{NH_3}} = \frac{[NH_3]\{H^+\}}{[NH_4^+]} \quad (6)$$

The right hand expression is called the "mixed acidity equilibrium constant" (Stumm and Morgan, 1970).

Let

$$K'a = \frac{Ka \cdot \{H_2O^n \cdot \gamma_{NH}\}_4^+}{\gamma_{NH_3}} \quad (7)$$

Taking the $\log_{10}$ of both sides and making the substitution that $pK = -\log K$, the following equation results:

$$pK'a = pKa - \log \gamma_{NH_4^+} + \log \gamma_{NH_3} - n \log \{H_2O\} \quad (8)$$

The values used for the right hand terms are as follows: $pKa = 9.154$ (Emerson *et al.,* 1975); $-\log \gamma_{NH_4^+} = 0.140$ (Stumm and Morgan, 1970; Whitfield, 1974); $\log \gamma_{NH_3} = 0.008$ (Whitfield, 1974); $-3 \log \{H_2O\} = 0.008$ (Robinson, 1954). The pK'a calculated for 28° C and 12‰ was 9.310 and was used in equation (1).

Effect of NH_3 and NH_4^+

To test the hypothesis that NH_3 is solely responsible for ammonia toxicity, the concentration of total ammonia was varied with pH to achieve equal levels of NH_3 but unequal levels of NH_4^+. As an example, using equation (1) and $pK'a = 9.31$, it is calculated that 10.3 mg ammonia/liter (9.3 mg NH_4^+/liter) will give 1.0 mg NH_3/liter at pH 8.34. But at pH 6.83, 303 mg ammonia/liter (302 mg NH_4^+/liter) is required for the same concentration of NH_3. Survival was monitored to learn if these widely divergent NH_4^+ concentrations affected larvae.

Results

Analysis of variance of mortality data showed no significant effect due to brood or any interaction involving brood and therefore, all data were combined for computation of LC_{50} and LT_{50} values. There was a highly significant effect ($P < 0.01$) due to both pH and ammonia concentration and the interaction of these variables. However, during the four bioassays performed (lethal and sublethal), survival of control larvae at each pH always exceeded 85% and averaged 95% for 144 to 168 hr exposures.

The toxicity of ammonia over a range of identical concentrations was greatly influenced by the pH of the media. The 24 hr LC_{50} values were 200, 115 and 37 mg ammonia/liter at pH 6.83, 7.60 and 8.34, respectively (Fig. 1), and the sensitivity to ammonia remained greatest at higher pH values throughout the tests. By 144 hr the LC_{50} values at the same pH values had decreased to 80, 44, and 14 mg/liter; approximately a 2.7 fold decrease from 24 hr values (Fig. 1). At the test's conclusion, slopes of toxicity curves for ammonia in solution at pH 8.34 and 7.60 were approaching asymptotes indicative of incipient LC_{50} values (Sprague,

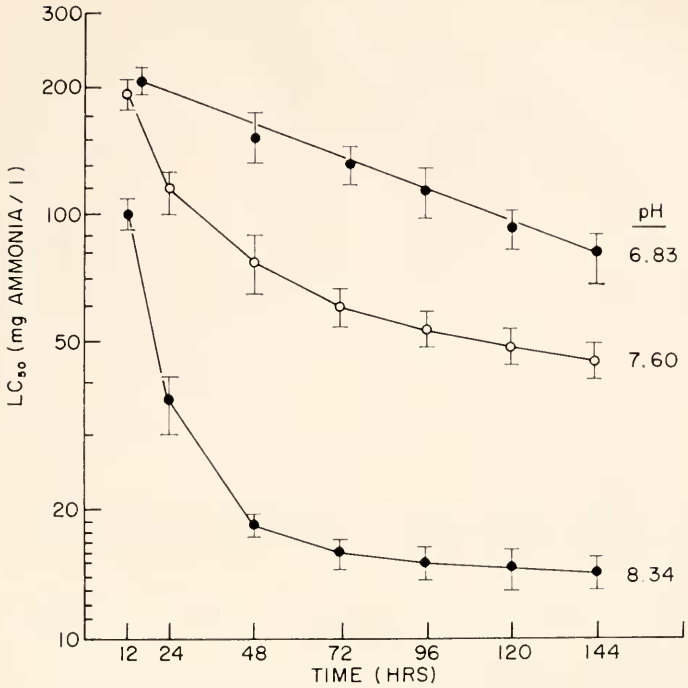

FIGURE 1. The toxicity of total ammonia to larval *M. rosenbergii* exposed in solutions of different pH. Bars are ± one standard deviation.

1969). However, no such decrease in the slope of the pH 6.83 toxicity curve had occurred, indicating longer tests were needed to estimate incipient concentrations.

The time to death for larvae held in solutions of equal ammonia concentration but different pH is shown in Figure 2. In 100 mg ammonia/liter, 50% mortality of larvae in solutions of pH 8.34, 7.60, and 6.83 occurred in about 9, 27, and 125 hr, respectively. Survival of larvae held in 32 mg/liter at pH 7.60 and 6.83 was nearly equal to that of controls. However, animals exposed to the same ammonia concentration at pH 8.34 were all dead by 48 hr (Fig. 2).

Un-ionized ammonia was not the exclusive toxic agent in these tests, and the NH_4^+ molecule apparently contributed to mortality also. When LC_{50} values were based on the concentration of NH_3 only (*i.e.,* normalized with respect to pH), there was no equality of the levels found to be toxic at specific time intervals (Fig. 3). In fact, larvae exposed to the lowest levels of NH_3 (pH = 6.83) were the most susceptible to toxicity due to the concomitantly high levels of NH_4^+ present (Table 1). The proportions of NH_3 and NH_4^+ found to be toxic were inversely related as pH changed. Consequently, about five times less NH_3 was lethal in a given period at low pH compared to the high, but it was accompanied by six times more NH_4^+ ion (Table 1).

The effect of NH_4^+ on survival times of larvae is further demonstrated when the response of groups in equal NH_3 concentrations at different pH values was compared. The LT_{50} values for larvae exposed to 0.98 mg NH_3/liter at pH 6.83

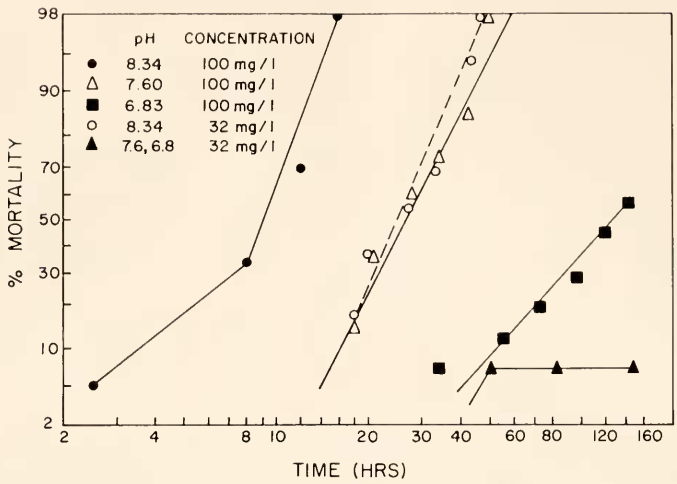

FIGURE 2. Cumulative percentage of mortality of *M. rosenbergii* larvae exposed to several combinations of ammonia and pH. Depicted are data for a single brood. Survival was adjusted to that of controls which averaged 95% at the end of an experiment.

and 8.34 were 9 hr and >144 hr, respectively, while the corresponding NH_4^+ concentrations were 319 and 9 mg/liter (Fig. 4). Animals exposed to 10.2 mg NH_3/liter survived twice as long as those exposed to 5.5 mg/liter, but the NH_4^+ concentration was 3.5 times higher in the latter case (Fig. 4).

Growth of *Macrobrachium* larvae was reduced in sublethal concentrations of ammonia and also seemed to be influenced by levels of NH_4^+ rather than NH_3. There was no significant effect of treatments in the first growth experiment. The initial mean weight was 52 ± 5 μg/larva and the final mean weight for all treatments was 77 ± 6 μg/larva, a 48% increase. In the second test, with smaller larvae exposed for a longer period, there was reduced gowth ($P < 0.01$) in solutions of 32 mg ammonia/liter at pH 6.83 and 7.60 (Table II). The initial weight of three-day old animals was 35 ± 6 μg each. At the test's conclusion, larvae of control groups weighed about 77 μg each (a 120% increase), while those in 32 mg/liter averaged 55 and 61 μg/larva (57% and 74% increases) in pH 6.83 and 7.60, respectively. These weights were significantly less than those of controls ($P < 0.05$,

TABLE I

Concentrations of ammonia toxic to M. rosenbergii *larvae expressed as both the NH_3 and NH_4^+ molecules.**

pH	24 hr LC_{50} (mg/liter)		144 hr LC_{50} (mg/liter)	
	NH_3	NH_4^+	NH_3	NH_4^+
6.83	0.66	199.34	0.26	79.74
7.60	2.10	112.90	0.80	43.20
8.34	3.58	33.42	1.35	12.65

* Total ammonia = $[NH_3] + [NH_4^+]$ and is depicted in Figure 1.

Q statistic) and were the only important differences found. Reduction in growth was not correlated with NH_3 concentrations. The relative growth rate (G_R) of controls was 0.108 g/(g dry body wt·day) (Table II). Larvae exposed to the highest NH_3 concentration of 0.98 mg NH_3/liter ($NH_4^+ = 9$ mg/liter) had a $G_R = 0.097$, while those exposed to 0.11 mg NH_3/liter ($NH_4^+ = 31.9$ mg/liter) had a $G_R = 0.063$ (Table II).

Discussion

The toxicity of ammonia to *Macrobrachium* larvae is inextricably linked to the pH of a solution, the total ammonia concentration present, and the proportions of that total which exist as either NH_3 or NH_4^+. Undoubtedly other factors, such as dissolved oxygen and salinity, could be varied from optimal levels to further complicate the story of ammonia toxicity to this crustacean.

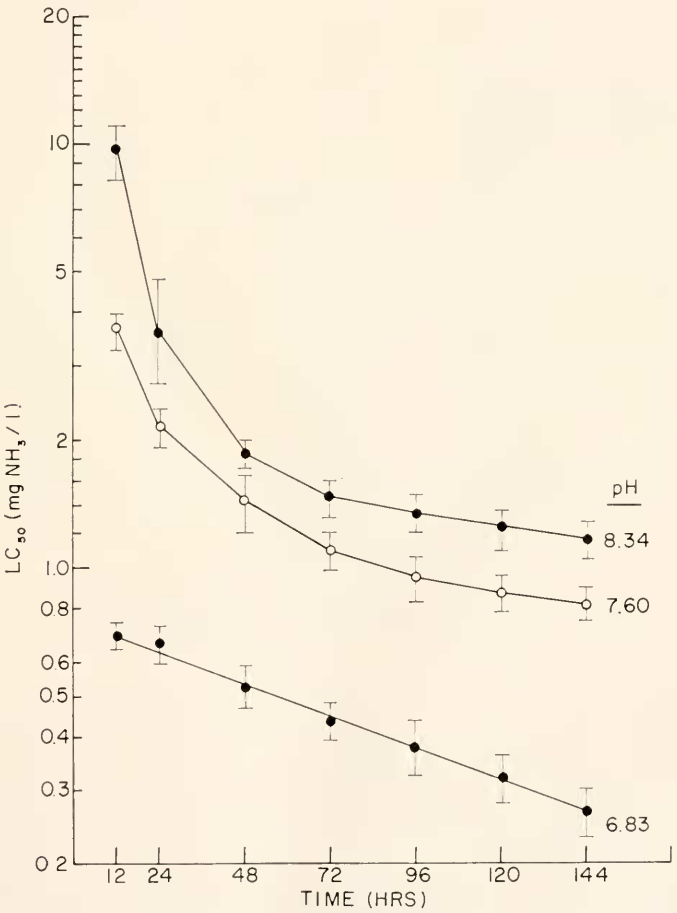

Figure 3. Concentrations of un-ionized ammonia (NH_3) causing 50% mortality in various time intervals. The NH_3 concentrations account for about 10%, 2%, and 0.3% of the total ammonia levels in the high to low pH values, respectively.

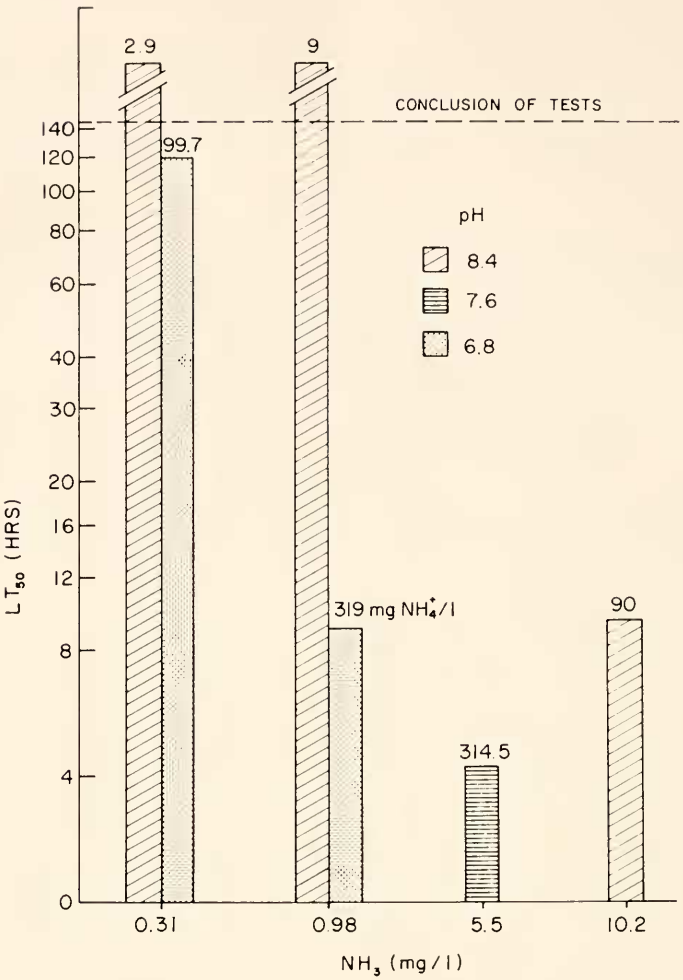

FIGURE 4. Time to 50% mortality of larvae exposed to several concentrations of NH_3 ammonia at different pH. At the top of bars are concentrations of NH_4^+. Bars exceeding 144 hr had survival equal to controls by the end of the experiment.

As is traditionally done in fish bioassays with ammonia, toxic concentrations derived from the present tests could not be normalized to pH variations by expressing results in terms of the NH_3 molecule only, because the NH_4^+ ion figured critically in causing stress. Toxic ammonia concentrations differ between the high and low end of the pH range tested, and are, we believe, determined by NH_3 at high pH and NH_4^+ at low pH values. At a pH of 8.34 the incipient LC_{50} value was estimated to be 14 mg ammonia/liter, which are NH_3 and NH_4^+ proportions of 1.35 and 12.65 mg/liter, respectively (Table I). Growth at this same pH was not inhibited by 10 mg ammonia/liter, indicating that an incipient lethal level is indeed about 12–14 mg/liter. In solutions of lower pH, more total ammonia is required

to cause toxicity, and the NH_3 fraction of these concentrations decreases exponentially with pH. Using growth as a sensitive gauge of stress, 32 mg ammonia/liter retarded development at both pH 6.83 and 7.60. The un-ionized NH_3 fraction at pH 6.83 is 0.11 mg NH_3/liter, only 0.3% of the total concentration and about 11 times less than the incipient LC_{50} value derived for pH 8.34. NH_4^+ ion accounts for nearly all ammonia present and is the species of ammonia probably responsible for toxicity at low pH values.

These observations may be combined in a model (Fig. 5) to describe differential ammonia toxicity caused by changes in pH. Water conditions shown in the model are those actually measured in these tests. Values for chemical factors in larval blood have been assumed based on literature data for adult and juvenile crustaceans. Blood osmolarity was estimated to be 500 mOsmol = 15.8‰ salinity based on determinations made with *M. rosenbergii* post-larvae (Armstrong and Nelson, unpublished data; Sandifer, Hopkins and Smith, 1975). Blood pH was chosen to be 7.55, 7.65, and 7.75 at corresponding water pH values of 6.83, 7.60, and 8.34 (from data of Johansen, Lenfant and Mecklenburg, 1970; Truchot, 1975; Weiland and Mangum, 1975; Mangum *et al.*, 1976). Total blood ammonia was taken to be representative of levels in control larvae, treated as described, before addition of toxic concentrations of ammonia to the ambient water. A concentration of 12 mg ammonia/liter of blood was assumed from data of Myers (1920), Florkin and Renwart (1939), Florkin and Frappez (1940), Gifford (1968), and Mangum *et al.* (1976). Sodium influx is depicted as relative magnitudes varying with ambient NH_4^+ concentrations. The pKa, 9.33, used to calculate un-ionized ammonia in the blood, was detemined for a salinity of 16‰, as previously outlined.

The model (Fig. 5) proposes that larvae exposed to ammonia at higher pH ($\approx$ 8.4) will be most affected by NH_3, which is nonpolar and can readily diffuse through biological membranes such as the gills (Warren, 1962). Of the total am-

TABLE II

Effect of ammonia on the relative growth rate of Macrobrachium *larvae held in water of different pH.*

pH	Total ammonia $NH_3 + NH_4^+$ (mg/liter)	Un-ionized ammonia NH_3 (mg/liter)	G_R* g/(g body wt·day)	Final mean** dry weight (±s.d.) µg/larva
6.83	0	0	0.107	77 (15)
	10	0.03	0.105	76 (11)
	32	0.11	0.063***	55 (11)
7.60	0	0	0.107	77 (16)
	10	0.20	0.113	81 (15)
	32	0.63	0.077***	61 (13)
8.34	0	0	0.109	78 (11)
	3.2	0.31	0.091	68 (12)
	10	0.98	0.097	71 (14)

* G_R = P/TM for dry wt. See text for explanation.

** Seven day exposure; initial mean dry weight = 35 ± 6 µg/larva; n = 19–23 larvae per group.

*** Significantly different from controls, $P < 0.05$.

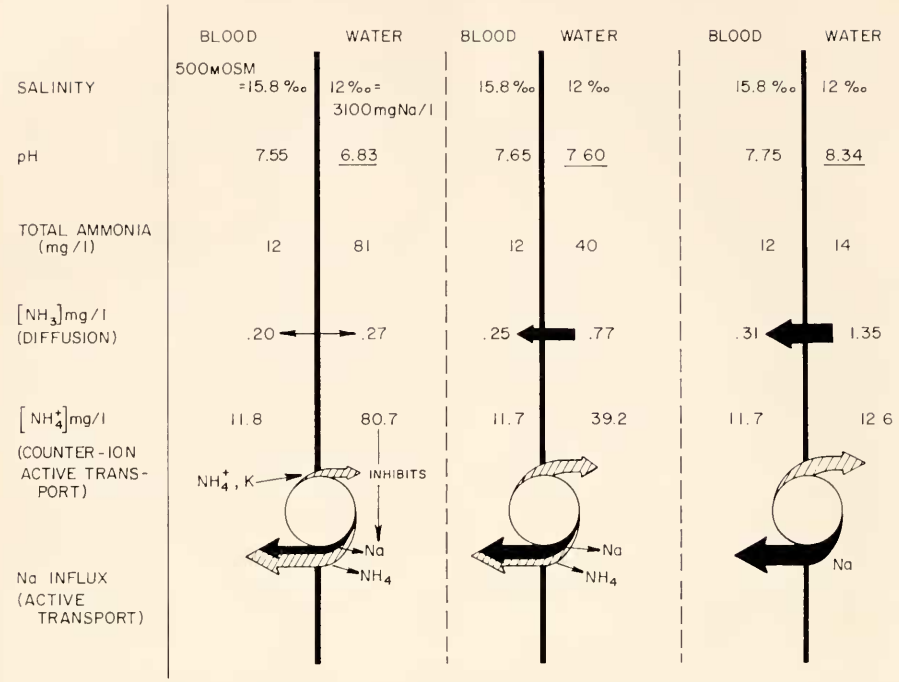

FIGURE 5. A proposed mechanism explaining the differential effects of NH_3 and NH_4^+ on *M. rosenbergii* larvae cultured in solutions of different pH. The values for blood salinity, pH, and total ammonia were estimated from literature data as described in the text. These conditions are assumed to be typical of larvae prior to addition of high ambient ammonia. The water ammonia levels are incipient lethal concentrations derived for the three pH values tested. Ammonia in water of high pH exists in relatively large quantities as unionized NH_3, which rapidly diffuses into larvae, increasing blood ammonia to toxic levels. In low pH solutions ammonia exists almost totally as NH_4^+. This ion is shown to compete with Na in active transport processes and toxicity ensues from osmoregulatory failure.

monia found toxic at high pH about 1.35 mg/liter or 10% exists as NH_3. This level exceeds that postulated for the blood by about four-fold, and consequently NH_3 would diffuse into animals. At a blood pH = 7.65 the molecule would be protonated to NH_4^+, thereby maintaining the NH_3 diffusion gradient inward. Body concentrations of ammonia would rise if alternate routes of excretion could not expel this surplus, and toxicity follow, perhaps *via* a mode described by Campbell (1973). Toxicity might include elevation of blood pH as NH_3 is protonated and a decrease in substrate for the tricarboxylic acid cycle as excess ammonia reverses the usual oxidation of glutamate (Campbell, 1973). Toxicity due to inward diffusion of NH_3 at high pH is rapid and caused mortality among test larvae in 2–18 hr (Fig. 2).

The deleterious effect of high ambient ammonia levels on an alternate route of ammonia excretion from the blood (nondiffusion) is the second component of the model. It is proposed that inhibition of sodium influx is a major factor contributing to ammonia toxicity at low pH. Larvae in water of pH 6.83 died in 81 mg

ammonia/liter, which is a NH_3 concentration of only 0.27 mg/liter. This water concentration is nearly equal to the blood level estimated and, even though the rate of diffusion of NH_3 outward is probably reduced, the decrease is apparently not serious. [Recall that 0.98 mg NH_3/liter at pH 8.34 caused no mortality (Fig. 4) or growth inhibition (Table II), yet this concentration certainly exceeded blood levels and should have established an NH_3 diffusion gradient inward.] Nearly all of the ammonia (80.7 mg/liter) exists as the NH_4^+ ion. By successfully competing with sodium ions, the NH_4^+ would both reduce the influx of Na^+, thereby diminishing body concentrations of this important salt, and also cause body levels of ammonia to rise by itself, riding the transport mechanism in or preventing metabolic NH_4^+ from riding it out. The resistance of the larvae to this form of osmoregulatory inhibition by NH_4^+ is apparently greater, and toxic manifestations do not develop as rapidly as when copious NH_3 diffusion inward (high pH) is operative. Mortality occurred in 40–140 hr at pH 6.83 (Fig. 2), and growth inhibition probably requires exposures of 5–7 days to be measurable with the larval stages used.

The hypothesis that toxicity at low pH is caused by inhibition of Na^+ transport by NH_4^+ (Fig. 5) has been based on several studies. Ammonium ion has long been suggested as a counter-ion for Na^+ transport (Krogh, 1939). Recently Mangum and Towle (1977) discussed the physiological roles of internal NH_4^+ in the euryhaline blue crab. They believe NH_4^+ aids in activating gill ATPase, serves as one counter-ion for sodium transport, aids in maintaining charge balance as it is excreted, and is an important form of ammonia in which this toxicant is eliminated from the body. In the external milieu, NH_4^+ can substantially reduce the influx of Na^+. Shaw (1960) found that 18 mg NH_4^+/liter caused an 80% decrease in Na^+ influx rates in the crayfish, *Astacus pallipes*. Inhibition of Na transport across gills by external NH_4^+ and stimulation of Na^+ uptake after intraperitoneal injection of NH_4^+ has also been documented for fish (Maetz and Garcia-Romeu, 1964; Carrier and Evans, 1976).

An interesting aspect of the Na^+–NH_4^+ transport system regards the affinity of the carrier mechanism for either molecule. Shaw (1960) found that the inhibition of sodium influx caused by ambient ammonium ion could be countered by increasing ambient sodium levels. Working with a freshwater crustacean in low levels of both Na^+ and NH_4^+, Shaw concluded that a concentration ratio of 10:1 favoring NH_4^+ must exist for inhibition of sodium transport to occur, and that the affinity of sodium for the transport site is greater than that of ammonium ion. The present experiments were done in 12‰ sea water or about 3100 mg Na^+/liter (Instant Ocean salt is 25.8% Na by weight based on manufacturer's analysis). Based on the concentrations of NH_4^+ found toxic (32–80 mg NH_4^+/liter), the NH_4^+ to Na^+ ratios in our tests were 0.01–0.02 : 1. Such low ratios for NH_4^+ imply that the ion has a greater affinity for the transport site than Na^+, contrary to Shaw's conclusion. This discrepancy might be partially explained by lower affinity of the transport mechanism for Na^+ in the euryhaline *Macrobrachium* than in the freshwater crayfish of Shaw's experiments. The K_m values for sodium transport may be tenfold greater in saline species than in similar freshwater forms (Prosser, 1973). Alternatively the low NH_4^+ : Na^+ ratios may indicate that NH_4^+ is causing toxicity in a manner other than inhibition of sodium movement.

It has been demonstrated in these studies that sufficiently high concentrations of NH_4^+ in water of low pH is lethal to crustacean larvae, even though the NH_3 concentration present may be sublethal. A model ascribes such toxicity to competitive inhibition of Na^+ transport. It is probably an over-simplification to attribute the toxicity of ammonia only to NH_3 at high pH and to NH_4^+ at low pH. There may be a contribution from each species at a total ammonia concentration found to be toxic, but we believe our model is accurate in assigning the bulk of toxicity to either NH_3 or NH_4^+ as the change in pH influences the ratios between them. Accordingly, we offer a caution for those studying ammonia-induced responses in organisms to consider the contribution from both NH_3 and NH_4^+ species in interpreting results. Relatively low but lethal concentrations of NH_3 may be accompanied by large amounts of NH_4^+, especially at lower pH values. High total ammonia levels used in some physiological experiments may represent near-lethal concentrations of NH_3, particularly at higher pH values. Mangum *et al.* (1976) reported that 10 mM = 180 mg ammonia/liter was used in tests on ammonia excretion. At a pH of about 7.8, this would equal 5.4 mg NH_3/liter, which is well within the range we found to be toxic (Table 1).

Finally, some discussion of the results relative to water quality requirements of crustaceans is warranted. Whether the maintenance of animals is for long periods in commercial operations or for short acclimations prior to physiological experiments, water quality is an important variable that should be monitored and regulated. Ammonia concentrations found to be toxic in this study are in accord with other values reported at similar pH levels. Wickins (1976) found that 101 mg ammonia/liter (pH = 7.0) gave an LT_{50} of 24 hr for adult *Macrobrachium*. Further, growth was reduced 30–35% in concentrations of 0.19–0.39 mg NH_3/liter, which corresponds to a very high range of 20–41 mg NH_4^+/liter (pH = 7.2, pKa = 9.22 at his test conditions). Following from the results of the present study, we suggest that inhibition of growth resulted primarily from the NH_4 ion and not NH_3, as reported (Wickins, 1976). Anderson (1944) reported that *Daphnia magna* was immobilized in 16–24 hr when exposed to 46 mg ammonia/liter (no pH given); and an incipient LC_{50} for larvae of the lobster, *Homarus americanus*, was 37 mg ammonia/liter at pH = 8.1, salinity = 33.4‰ (Delistraty *et al.*, 1977). The incipient LC_{50} calculated for *Macrobrachium* larvae in water of pH 7.60 was 40 mg ammonia/liter.

These toxic concentrations are rather high and greatly exceed the "safe" level of 0.1 mg ammonia/liter recommended by Spotte (1970). Larvae in the present test survived 10 and < 32 mg ammonia/liter for seven days at pH = 8.34 and 6.83, respectively. Such levels would probably be injurious over long periods and an application factor, applied to the incipient LC_{50} values or concentrations inhibiting growth, would be needed to estimate safe levels. Sprague (1971) summarizes thought on this topic with the conclusion that 0.1–0.3 of an incipient LC_{50} value can predict safe concentrations. Such a criterion would predict as safe about 1 mg ammonia/liter at pH 8.34 and 3.2 mg/liter at the lower pH. However, the lack of mortality and sublethal growth inhibition at 10 mg/liter leads us to conclude that short-term exposure to rather high ammonia levels may not be damaging to *Macrobrachium*.

In general, the use of flow-through culture systems with water exchange ade-

quate to dilute excreted ammonia, or closed-systems with conditioned, nitrifying filters for detoxification should minimize the threat of ammonia toxicity for crustaceans. In our research culture facilities, the ammonia concentration in water passed through biological filters averages 0.5 mg/liter (pH $\approx$ 8.1), well below toxic levels reported in this study.

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Summary

1. The toxicity of ammonia to *Macrobrachium* larvae was tested at pH 6.83, 7.60, and 8.34, and the respective 144 hr LC_{50} values were 80, 44, and 14 mg ammonia/liter.

2. Toxicity of ammonia was not due solely to the NH_3 molecule. In solutions of different pH and equal NH_3 concentrations, survival was greatly reduced as NH_4^+ levels increased.

3. A model is proposed to explain the differential effect of ammonia as pH varies. At higher pH (8.4) toxicity results from copious diffusion of NH_3 into larvae. At lower pH (6.8) toxicity is thought to result from competitive inhibition of Na^+ transport by NH_4^+.

4. Retardation of growth was documented in sublethal concentrations of ammonia at 6.8 and 7.6. The average dry weight was about 26% less than that of controls ($P < 0.05$) after a seven day exposure.

5. Results are discussed relevant to the culture and maintenance of crustaceans, and it is concluded that ammonia will not pose a substantial threat in adequately managed systems.

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